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(54) Title: AGENTS FOR THE DIAGNOSIS AND TREATMENT OF TUMOURS THAT EXPOSE ALTERED PROTEINS ON THE CELL SURFACE

(57) Abstract: The present invention relates to agents for the diagnosis and treatment of tumours that expose altered proteins on the cell surface.

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AGENTS FOR THE DIAGNOSIS AND TREATMENT OF TUMOURS
THAT EXPOSE ALTERED PROTEINS ON THE CELL SURFACE

The present invention relates to agents for the diagnosis and treatment of tumours that expose altered proteins on the cell surface.

BACKGROUND OF THE INVENTION

Some tumours expose on the cell surface proteins structurally altered as
5 a result of somatic mutations. Tumours may expose structurally altered proteins also as a result of splicing variations, altered post-translational modification or partial degradation.

One of the most frequently studied families of altered proteins exposed on the surface of tumour cells derives from E-cadherin, a calcium-dependent
10 cell adhesion molecule firmly anchored in the cytoplasmic membrane. More than 33 distinct somatically mutated forms of E-cadherin have been identified in infiltrative lobular breast cancer (Berx et. al., *Hum. Mutat.* 12: 226-237, 1998; Becker et al., *Hum. Mutat.* 13: 171, 1999). Most of these mutated forms are truncated proteins resulting from out of frame deletion mutations.
15 Normally tumors in each patient only display one particular mutated form of E-cadherin

Human gastric tumours of the diffuse type have been described to frequently express somatically mutated E-cadherins. In this tumour, besides point mutations leading to the replacement of single amino acids, the
20 mutations often involve an exon-skipping in-frame deletion, leading to a minimally shortened and regionally altered amino acid sequence. Such in-frame deletions have been observed in correspondence of at least 9 of the 16 exons in the E-cadherin gene. Deletions at exon 8 or 9 are by far the most frequent, followed by deletions at exon 10 and 7. These mutations are specific
25 for tumour cells, and are never present in healthy cells; they consequently

constitute an ideal target for immunotherapeutic approaches. Identification of the particular type of mutated E-cadherin present in the tumours of a patient, requires corresponding immunodiagnostic approaches.

US 6,447,776 and EP0821060 A2 disclose monoclonal antibodies which
5 specifically recognise mutated forms of E-cadherins. They also disclose a diagnostic or therapeutic agent in which one of these antibodies (recognition unit) is conjugated with a diagnostic radiation source (diagnostic-signal-generating unit), a therapeutic radiation source (therapeutic effect-generating unit) or a toxin (therapeutic effect generating unit). Mixtures of at least two of
10 the disclosed agents are claimed.

Application of a product in which the therapeutic-effect-generating unit was the alpha particle-emitting radioisotope, ^{213}Bi , used for locoregional radioimmunotherapy of murine tumours expressing altered E-cadherins, was described by Senekowitsch-Schmidtke et al. in "Ninth Conference on Cancer
15 Therapy with Antibodies and Immunoconjugates", Abstract 21, Oct. 24-26, 2002, Princeton, New Jersey. Some of the same authors, in an earlier paper (Becker et al., "Molecular Targets and Cancer Therapeutics", Miami Beach, Florida, 29 October-2 November 2001), proposed the use of a conjugate of a cytotoxic agent (toxin) with a monoclonal antibody able to recognise a
20 particular E-cadherin mutant for personalised treatment of patients suffering from tumours characterised by that somatic mutation.

These approaches, which require the preparation of a distinct product for each particular mutation found in a population of patients, may be promising in some cases, but is limited by the cost problem associated with
25 the development and production of multiple personalised drugs, i.e. a separate drug for as many types of E-cadherin mutations as one would like to be able to diagnose and/or treat therapeutically. In principle, administration of a mixture of such products targeted to all, or at least the majority of possible mutated E-

cadherins, as claimed in US6447776, would partially get around the cost problem. However, this solution for the cost problem is not acceptable from a safety risk point of view, because products in the mixture not specific for the mutated E-cadherin on the patient's tumour cells would involve a toxicological overload, i.e. exposure to radiation or exposure to cytotoxic agent, not justified by therapeutic or diagnostic benefits; these drawbacks would prevent regulatory approval of the mixture of agents.

These arguments about costs and safety risks are equally applicable to cases other than that of E-cadherin, including cases in which the various forms of structurally altered tumour surface proteins have their origin in altered splicing, post-translational modifications or altered degradation. An example of altered post-translational modification are incomplete glycosylation as a result of altered synthesis or as a result of partial degradation, provided not all altered forms occur simultaneously in each patient. Examples of alterations due to partial degradation derive from a small number of proteolytic cleavages, typically a single cleavage, inside the amino acid sequence of the extracellular domain of a membrane protein.

The above arguments are also equally applicable to targeted agents with a diagnostic-signal-generating or therapeutic-effect-generating unit of a different kind including, without thereby limiting the possibilities, radioactive halogen atoms, chelates of α , β - or γ - emitting radioisotopes, chelates of paramagnetic metal ions, chromophores for photodynamic therapy, and cytotoxic compounds.

The present invention offers a solution to the safety risk and the cost problem. The solution involves a special polyspecific targeting agent.

Polyspecific targeting agents are agents that are capable of binding to more than one structurally distinct molecular target site. Such agents are well known in the art and can be prepared by many different methods, as

summarized in US2002/0025317 A1. In short, polyspecificity can be achieved by covalently or non-covalently conjugating or biochemically fusing elements that on their own show specific binding to distinct target sites. A particular form of bispecific agent is the bispecific antibody or its F(ab')₂ fragment, a so-called diabody. In this case heavy and light chains of two antibodies with distinct specificities are combined into a hybrid structure that recognizes with each of its halves the distinct target sites, instead of recognizing, like in a normal antibody the same target site with two separate arms.

10 There exist polyspecific targeting agents of the first kind that are designed to recognize with at least one of their specificities a biological target in vivo, and with at least one other of their specificities, another molecule artificially introduced into the body. Polyspecific targeting agents of the second kind are designed to recognize multiple natural targets in vivo. Here
15 with polyspecific targeting agents those of the second kind are meant, without thereby excluding combinations of the first and second kind.

 The polyspecific targeting agents of the art have one of the following properties:

 Polyspecific targeting agents of the art recognizing different target sites
20 on the same target molecule have increased avidity and specificity of the agent for its target.

 Polyspecific targeting agents of the art recognizing distinct target sites on different molecules on the same cell have increased specificity and capacity of binding to cells that display simultaneously both targets or achieve
25 additivity or synergy in action on both targets, thereby increasing the efficacy achievable with the polyspecific agent over the one achievable with a monospecific agent.

 Polyspecific targeting agents of the art recognizing target sites on

distinct molecules on different cell types simultaneously present in the tissue, achieve additivity or synergy between the binding and biological effects to the different cell types.

A common characteristic of all polyspecific targeting agents of the art and their applications is the interaction of the agent with all the multiple simultaneously present target sites for which they possess specificity. The advantages of polyspecificity over monospecificity in products of the art are intrinsically linked to the availability of all the multiple distinct target sites in the same patient.

The polyspecific agent of the present invention shares with the polyspecific agent of the art the basic construction as a conjugate, covalent or not, of a polyspecific recognition unit, composed of at least two recognition molecules, and a diagnostic-signal-generating or therapeutic-effect-generating unit. However, the polyspecific agent of the present invention is distinguished from the polyspecific agent of the art by the following characteristics:

Whereas the polyspecific agent of the art possesses specificities matched in number to the number of corresponding distinct types of target sites simultaneously present in a given patient, the polyspecific agent of the present invention possesses more distinct specificities than there are corresponding distinct types of target sites in any one patient.

Whereas the polyspecific agent of the art interacts in all patient with the same combination of distinct types of target sites, the polyspecific agent of the present invention does not interact in all patients with the same combination.

Whereas the polyspecific agent of the art profits in terms of overall specificity and avidity of the diagnostic or therapeutic agent in any given patient from the presence of all the specificities, the polyspecific agent of the present invention profits in any given patient only from the presence of a subset of all available specificities.

The differences between agent of the art and agent of the present invention is particularly pronounced in the special but most useful case, where each patient displays only a single abnormal protein (e.g. the case of E-cadherin) and the polyspecific agent of the invention utilizes in each patient
5 only a single specificity from among its multiple ones. In this case multispecificity makes no contribution to increased specificity and avidity of polyspecific agent over monospecific analogue.

The present invention embodies the surprising realization that a diagnostic or therapeutic agent of the invention, i.e. a polyspecific targeting
10 agent with N distinct specificities is advantageous

- a) with respect to a mixture of N monospecific agents in terms of the risk to the patient, when it utilizes only a number smaller than N of its N specificities, especially when it utilizes only a single of its N specificities, in any given patient.
- 15 b) with respect to N separate monospecific agents in terms of drug development and production costs even when it utilizes only a single of its multiple specificities in any given patient.

A risk-related advantage of the product of the invention to the patient arises provided the following three conditions are met simultaneously:

- 20 1) The polyspecific recognition unit possesses N distinct target specificities, each specific for another of the various altered forms that a given protein in a tumour subtype can assume in a population of patients.
- 2) Each patient displays on its tumour, among the N altered forms of the protein recognized by the polyspecific recognition unit, only a number
25 smaller than N of them, typically a single one.
- 3) The diagnostic-signal-generating unit or therapeutic-effect-generating unit has some toxic effects on or constitutes a risk to healthy tissue or the organism as a whole, radiation exposure being included in such risks.

DESCRIPTION OF THE INVENTION

The first aspect of the invention relates to an agent for the diagnosis or treatment of tumours that in an individual patient exposes on the cell surface only a subset of the different, characteristic altered forms that a given protein
5 of said tumour can take, said protein deriving from alterations of a normal form present in healthy tissue, said agent comprising:

- a. a polyspecific recognition unit consisting of a recognition molecule specific for a first of said altered forms of the protein, conjugated with at least one other recognition molecule which
10 recognises a different of said altered forms of the same protein not simultaneously present on the tumour;
- b. at least one diagnostic-signal-generating or therapeutic-effect generating unit which supplies a diagnostic signal or therapeutic effect, conjugated with or included in said polyspecific
15 recognition unit.

The invention also relates to diagnostic or pharmaceutical compositions containing a polyspecific agent as defined above, in admixture with a suitable vehicle.

DETAILED DESCRIPTION OF THE INVENTION

20 The term "altered protein" means a protein with a structural alteration or modification, as will be specified in detail below.

Antibodies or fragments thereof able to recognise and specifically bind the altered proteins expressed by tumours can be used as recognition molecule according to the invention.

25 Fab, Fab', F(ab')₂ or scFv antibody fragments and derivatives are particularly preferred. Diabodies and their derivatives are also preferred. Alternatively, polypeptides, proteins, polysaccharides or other molecules with affinity for said altered proteins can be used.

These recognition molecules can be conjugated by chemical methods, using conventional polyfunctional reagents commonly employed in the field. The same methods can be used to chemically conjugate the recognition molecules or the entire polyspecific recognition unit with the diagnostic-
5 signal-generating or therapeutic-effect-generating unit. Alternatively, the diagnostic-signal-generating or therapeutic-effect-generating unit may be conjugated to one of the recognition molecules by expression of genes fused by recombinant DNA techniques. For example a the gene for a proteic toxin may be fused with the gene of one of the two genes expressing the light or the
10 heavy chain of immunoglobulin Fab fragments. Polyspecific recognition units can also be constructed fusing genes coding for multiple scFv through suitable linkers.

A special case of a polyspecific recognition unit suitable for the construction of agents of this invention is the diabody, in which the
15 conjugation chemistry between recognition units with distinct specificities is based on the spontaneous reformation of disulfide bridges in orthologous positions during reoxydation of a mixture of two partially reduced antibodies or F(ab')₂ fragments with different specificities. Preparation of diabodies is well known art (EP404097; WO93/11161; Hollinger et al., Proc. Natl. Acad.
20 Sci. USA 90: 6444-6448,1993). Diabodies or their F(ab')₂ fragments by themselves can serve as recognition unit of the invention, or they can be used as individual recognition molecules of a larger polyspecific recognition unit.

The altered proteins expressed or exposed by tumours which can be recognised by the agents according to the invention typically present one or
25 more mutations, point mutations, deletions, insertions or truncations, absence of post-translational modifications, altered post-translational modifications or effects of partial degradation.

Preferred examples of said altered proteins are the proteins known as E-

cadherins, which in diffuse gastric tumours often present with in-frame deletions in various exons, deletions that are frequently accompanied by the creation of novel antigenic sequences of amino acids. A preferred agent according to the invention is therefore constituted by a polyspecific agent
5 composed of a first monoclonal antibody or a fragment or derivative thereof which recognizes E-cadherin with deletion mutation in exon 8, conjugated to second monoclonal antibody or fragment or derivative thereof which recognizes E-cadherin with a deletion mutation in exon 9, and further conjugated to a diagnostic-signal-generating or therapeutic-effect-generating
10 unit of the kind specified below.

Said diagnostic-signal-generating or therapeutic-effect-generating unit can be covalently bound directly, or through a suitable linker, to one of the recognition molecules of the polyspecific recognition unit. Alternatively it may be covalently bound to the linker between the multiple recognition
15 molecules or it may be integral part of the linker.

In another embodiment of the invention the diagnostic-signal-generating or therapeutic-effect-generating unit can be conjugated covalently with biotin, in which case the polyspecific recognition unit will be conjugated covalently with avidin or streptavidin. Alternatively, the diagnostic-signal-
20 generating or therapeutic-effect-generating unit can be conjugated covalently with avidin or streptavidin, in which case the polyspecific recognition unit will be conjugated covalently with biotin.

Covalent conjugation between the multiple recognition molecules and between the polyspecific recognition unit and the diagnostic-signal-generating
25 and therapeutic-effect-generating unit is preferably obtained by reactions involving free sulfhydryl groups naturally present or generated by partial reduction of available disulfide bridges. The reagents are preferably selected from among compounds having one of the following residues: maleimino,

iodoacetyl, 2,4-dinitro-fluorophenyl, pentafluorophenyl. Linkers containing multiple maleimide groups capable of reacting with free sulfhydryl groups, thereby allowing the conjugation of recognition molecules among themselves and their conjugation with the diagnostic-signal-generating or therapeutic-effect-generating unit, as well as reaction conditions for achieving conjugation, have been described for example in Smith BJ et al.: *Bioconjugate Chem.* **12**, 750-756, 2001. However, the covalent conjugation required by the present invention can also be achieved with chemistry involving other functional groups on the various components, such as OH, -NH₂ and -COOH groups, using chemistry well known in the art.

As the diagnostic-signal-generating or therapeutic-effect-generating unit can be designed to contain several of said functional groups, it can itself act as linker between the specific recognition molecules.

The diagnostic-signal-generating or therapeutic-effect-generating unit can be selected from among radioactive halogens, chelates of radioactive isotopes or paramagnetic metal ions, particles of iron oxide, stabilised microbubbles, fluorescent or phosphorescent compounds, near-infrared radiation-absorbing compounds, cytotoxic compounds, toxins, or photodynamic compounds able to generate reduced oxygen species or singlet oxygen species by irradiation, without thereby limiting the scope of the invention.

The radioactive isotope is preferably selected from among halogen isotopes ¹²³I, ¹²⁴I, ¹²⁵I, ¹³¹I, ⁷⁵Br, ⁷⁶Br, ⁷⁷Br and ⁸²Br or radioactive isotopes of other elements such as ^{99m}Tc, ¹¹¹In, ²⁰³Pb, ⁶⁶Ga, ⁶⁷Ga, ⁶⁸Ga, ¹⁶¹Tb, ⁷²As, ^{113m}In, ⁹⁷Ru, ⁶²Cu, ⁶⁴Cu, ⁶⁷Cu, ⁵²Fe, ^{52m}Mn, ⁵¹Cr, ¹⁸⁶Re, ¹⁸⁸Re, ⁷⁷As, ⁹⁰Y, ¹⁶⁹Er, ¹²¹Sn, ¹²⁷Te, ¹⁴²Pr, ¹⁴³Pr, ¹⁹⁸Au, ¹⁹⁹Au, ¹⁰⁹Pd, ¹⁶⁵Dy, ¹⁴⁹Pm, ¹⁵¹Pm, ¹⁵³Sm, ¹⁵⁷Gd, ¹⁵⁹Gd, ¹⁶⁶Ho, ¹⁷²Tm, ¹⁶⁹Yb, ¹⁷⁵Yb, ¹⁷⁷Lu, ¹⁰⁵Rh, ¹¹¹Ag, ⁴⁷Sc, ¹⁴⁰La, ²¹²Bi, ²¹¹At, ²¹³Bi, ²¹²Pb, ²²⁵Ac, ²²³Ra, ²²⁴Ra and ²²⁷Th. In some cases the same

isotope allows diagnosis and treatment. For diagnostic applications, in particular Magnetic Resonance Imaging (MRI) techniques, a chelate of a paramagnetic metal selected from among the metal elements having an atomic number of 21-29, 39, 42, 44, 49 or 57-83 will be used. Chelates of the metal
5 ions Gd^{3+} , Fe^{3+} , Eu^{3+} , Dy^{3+} , La^{3+} , Yb^{3+} and Mn^{2+} are preferred.

Chelating groups are chosen from among the large number described in the art to be suitable for imaging or radiotherapy with the chosen metal ion and/or isotope when conjugated to a targeting agent. Obviously also polyspecific agents of the presently described kind containing novel chelating
10 groups fall within the scope of the present invention.

Chelating groups can be conjugated to the recognition molecule either directly or by means of reactive groups such as maleimide, bis-maleimide, lysine residues and the like.

Examples of cytotoxic compounds are also residues of known
15 antitumoral compounds, in particular residues with alkylating activity such as cyclophosphamide, chlorambucil, or natural or synthetic toxins.

For the proposed therapeutic and diagnostic uses, the agents according to the invention will be suitably formulated in the form of compositions in admixture with an appropriate vehicle.

20 The doses can be determined by skilled persons in the field on the basis of the pharmacokinetic and toxicological characteristics of the selected agent, as well as the type of application involved. Established guidelines which aid determination of the dose by analogy with the immunoconjugates and paramagnetic contrast agents already available for therapeutic and diagnostic
25 applications are also available. For example, when the necessary quantity of ion, radioactive compound or paramagnetic metal has been determined, the quantity of the agent according to the invention can be determined by means of a simple stoichiometric calculation. The compositions according to the

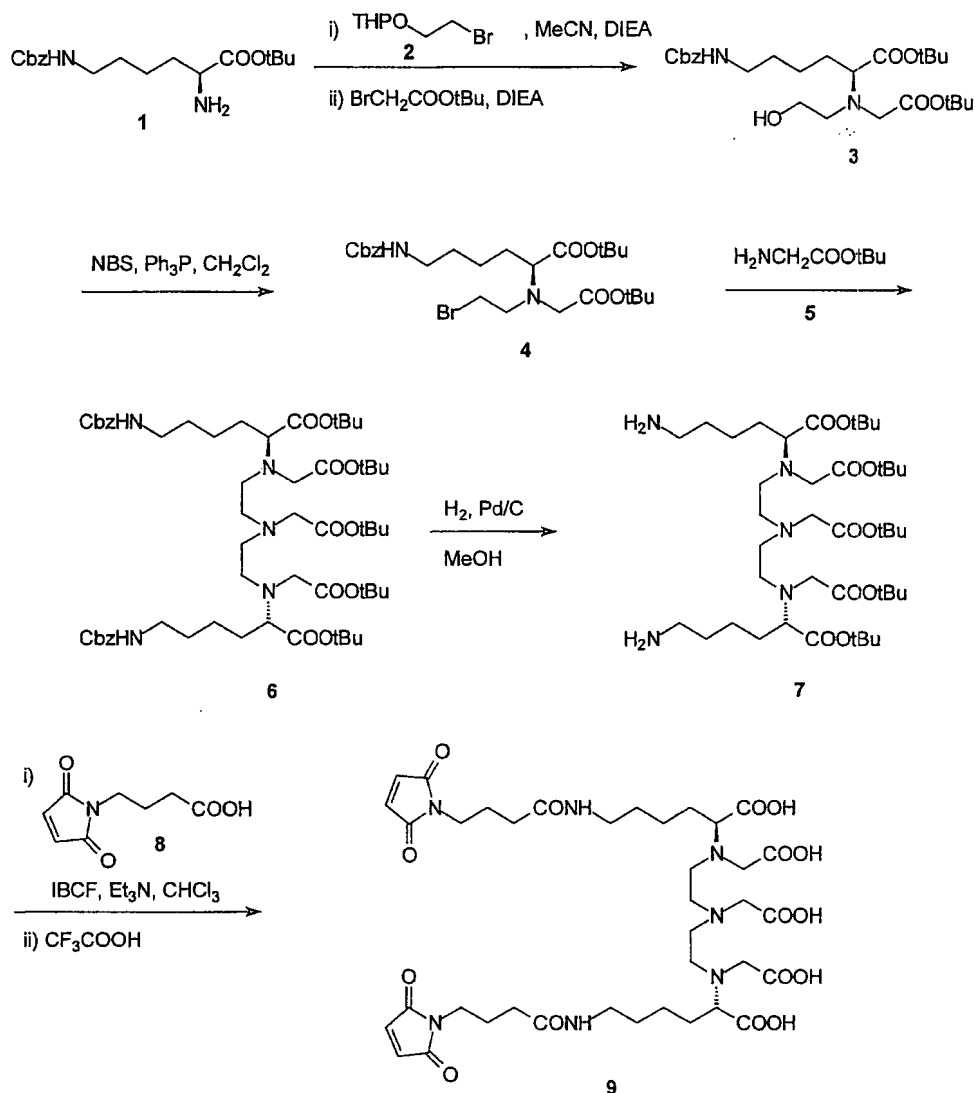
invention will preferably be in the form of solutions or suspensions in sterile vehicles suitable for parenteral administration, in particular intravenous, intraperitoneal or intramuscular administration.

The compositions according to the invention may also be supplied in
5 the form of kits comprising:

- a. the unit able to provide a diagnostic signal or therapeutic effect, covalently conjugated with biotin, and
- b. the recognition unit covalently conjugated with avidin or streptavidin or, alternatively,
- 10 c. the unit able to provide a diagnostic signal or therapeutic effect, covalently conjugated with avidin or streptavidin, and
- d. a recognition unit covalently conjugated with biotin.

In this case, separate administration of components a and b will allow *in vivo* formation of the agent according to the invention.

15 The following examples illustrate the invention in greater detail.

Example 1**Synthesis of a bis(maleimide) derivative of DTPA (Compound 9).****Scheme**

5

Compound 3

A solution of N^ε-[(phenylmethoxy)carbonyl]-L-lysine *t*-butyl ester (compound 1) (prepared according to *Bioconjugate Chem.* 10: 137-140, 1999) (100 mmol), 2-(2-bromoethoxy)tetrahydropyran (compound 2) (prepared according to *J. Org. Chem.* 51: 752-755, 1986) (135 mmol) and diisopylethylamine (100 mmol) in MeCN is maintained under reflux for 14

h. *t*-butyl-bromoacetate (120 mmol) and more diisopropylethylamine (100 mmol) are added, and the mixture is maintained under reflux for a further 2 h. The solution is then evaporated to give a residue which is dissolved in Et₂O and washed with water, 1 N HCl, 1 N NaOH and water. The solution is
5 evaporated, the residue is re-dissolved in MeOH, and 2 N HCl is added. After agitation for 2 h, 2 N NaOH is added until pH 7 is reached, then the solution is evaporated to eliminate the MeOH, and Et₂O is added to extract the product. The organic solution is separated, dried over Na₂SO₄ and evaporated to give crude compound 3, which is purified by flash chromatography.

10 The ¹H-NMR, ¹³C-NMR, MS and IR spectra proved consistent with the structure indicated.

Compound 4

N-Bromosuccinimide (52 mmol), in portions, is added to a solution of compound 3 (40 mmol) and triphenylphosphine (52 mmol) in CH₂Cl₂ cooled
15 to 0°C, under stirring. The temperature of the solution is allowed to rise to room temperature, and it is washed after 4 h with water, 5% NaHCO₃ and water. The organic solution is dried (Na₂SO₄) and evaporated. The residue is purified by flash chromatography to give compound 4.

The ¹H-NMR, ¹³C-NMR, MS and IR spectra proved consistent with the
20 structure indicated.

Compound 6

A biphasic mixture of compound 4 (22 mmol) and glycine *t*-butyl ester hydrochloride (compound 5) (commercial product) (10.4 mmol) in MeCN and 2 M phosphate buffer at pH 8 is stirred vigorously. After 24 h the two phases
25 are separated and the aqueous phase is replaced by fresh 2 M phosphate buffer. After stirring for a further 24 h, the organic phase is separated and evaporated. The residue is purified by flash chromatography to give compound 6.

The ^1H -NMR, ^{13}C -NMR, MS and IR spectra proved consistent with the structure indicated.

Compound 7

Pd/C (10%) is added to a solution of compound 6 in methanol, and the
5 suspension is agitated for 6 h in a hydrogen atmosphere (1 atm; 20°C). The resulting mixture is filtered and evaporated to give compound 7.

The ^1H -NMR, ^{13}C -NMR, MS and IR spectra proved consistent with the structure indicated.

Compound 9

10 Isobutyl chloroformate (13 mmol) is added dropwise, under agitation, to a solution of 4-maleimidobutyric acid (compound 8) (12 mmol) and Et_3N (13 mmol) in THF at -15°C under a hydrogen atmosphere. A solution of compound 7 (5 mmol) in THF is added dropwise after 30 min. After a further 30 min at -15°C, the temperature of the reaction mixture is allowed to rise to
15 room temperature, and agitation is continued for 4 h. The solution is then evaporated and the residue dissolved in EtOAc and washed with water. The organic phase is dried (Na_2SO_4) and evaporated. The residue is dissolved in CH_2Cl_2 , and CF_3COOH (100 mmol) is added. After 16 h the solution is evaporated, the residue is taken up with fresh CF_3COOH , and the resulting
20 solution is kept under stirring for a further 6 h. The solution is then evaporated and the residue is purified by through elution on a resin (Amberlite® XAD 16.00T) with an MeCN/water gradient. The fractions containing the pure product are combined and evaporated to give compound 9.

The ^1H -NMR, ^{13}C -NMR, MS and IR spectra proved consistent with the
25 structure indicated.

Example 2

Conjugation of two different Fab fragments with a single molecule of compound 9 (Compound Fab1-c9-Fab2)

A volume, V , of a 2 mM solution of tris-carboxyethylphosphine (TCEP) is prepared by 1 to 250 dilution of the 0.5 M commercial product (Pierce) in a thoroughly de-aerated pH = 7 buffer containing 50 mM Tris-HCl and 5 mM EDTA. This solution is then added to an equivalent volume, V , of a 10 μ M solution of a first human anti-*Herpes simplex* recombinant Fab fragment (Fab1), prepared according to Cattani et al. (*J. Clin. Microbiol.* 35: 1504.1509, 1997) and incubated for 30 min at 37°C. Half a volume ($V/2$) of a 50 mM solution of compound 9 in 0.1 M acetate buffer at pH = 5 is then added, and the reaction mixture is maintained at 37°C for 1 h. The reaction is then complete, and the surplus reagents is removed with conventional separation technologies such as dialysis or gel filtration.

For analysis purposes, a sample is injected into a TSK-G2000SW-XL size exclusion column, and this allows the demonstration that the majority of the protein remains approximately the size of a Fab fragment. Only a small part is approximately the size of two Fab fragments. The product which has the same size as one Fab is purified on a Sephacryl S-200HR size-exclusion column (Amersham Biosciences). The recovered material is further purified on a cation exchange column (Resource-S, Amersham Biosciences) and eluted with a saline gradient. The peak corresponding to the 1:1 conjugate of Fab1 with compound 9 (Fab1-c9) is collected and set aside.

A volume, V , of a 2 mM solution of TCEP is prepared by 1 to 250 dilution of the 0.5 M commercial product (Pierce) in a thoroughly de-aerated pH = 7 buffer containing 50 mM Tris-HCl and 5 mM EDTA. This solution is added to an equivalent volume, V , of a 10 μ M solution of a second Fab fragment (Fab2), specific for tetanus toxin, isolated by digestion with papain

of a commercial antibody (Terbutalin, Baxter AG, Vienna), and incubated for 30 min at 37°C, yielding the reduced Fab2.

A molar quantity of Fab1-c9 equivalent to that of the reduced Fab2 is then added as 10 µM solution in 0.1 M acetate buffer at pH = 5, and the
5 reaction mixture is maintained at 37°C for 1 h.

The reaction mixture is separated on a Sephacryl S-200HR size-exclusion column, and material of a size approximately equivalent to two Fab fragments is isolated. The final material, called Fab1-c9-Fab2, is proven to be homogeneous when tested on a TSK G2000SW-XL analytical size-exclusion
10 column.

Example 3

Conjugate with two different anti-mutated E-cadherin Fab fragments (compound Fab3-c9-Fab4)

Fab fragments of rat antibody fully specific for E-cadherins with
15 mutation in both exon 8 (Fab3) and exon 9 (Fab4) are prepared according to the method of Becker et al. (Poster #648, Molecular Targets and Cancer Therapeutics. Miami Beach, Florida, Oct. 29-Nov. 2, 2001). These Fabs do not interact with natural E-cadherins. The Fab3-c9-Fab4 conjugate is prepared according to the teaching of example 2.

Example 4

Labelling of Fab1-c9-Fab2 with ¹¹¹In

The conjugate described in Example 2, Fab1-c9-Fab2, is formulated at the concentration of 0.25 mg/mL in pH 6 acetate buffer. The Indium-111 chloride is available from Amersham at the concentration of 0.2 µg/mL
25 (10 mCi/mL). Labelling is performed by incubation at room temperature for 30 min. Labelling efficiency is tested by thin-layer chromatography with ITLC-SG strips (Gelman Laboratories), using an 0.9% solution of NaCl as mobile phase.

The reaction mixture is also analysed through HPLC by size-exclusion chromatography with a TSK-gel G3000 column; phosphate-buffered saline (PBS) added with 0.2 M NaCl was used as eluent. The eluate was monitored by UV detector at the wavelengths of 280 and 254 nm and a radiometric
5 detector placed in series with the UV detector. The radiopharmaceutical, ^{111}In -Fab1-c9-Fab2, gives a single radioactivity peak corresponding to the unlabelled protein. 98% labelling efficiency is obtained with a Fab1-c9-Fab2/ $^{111}\text{InCl}_3$ stoichiometric molar ratio of 3/1.

Example 5

10 Labelling of Fab3-c9-Fab4 with ^{177}Lu

Using the conjugate Fab3-c9-Fab4 and lutetium-177 chloride in molar proportions 1:0.9, the procedure described in example 4 supplies a conjugate labelled with lutetium-177. The product can be used in radioimmunotherapy of metastases deriving from stomach tumours that bear E-cadherin with a
15 mutation deletion in either exon 8 or exon 9, but never bear both mutated E-cadherins simultaneously or both mutations in the same E-cadherin. The same product may be used for both cases without any disadvantage in terms of radiation dose compared with a product with a single specificity for one or the other of the mutated E-cadherins, and with a net advantage in terms of
20 radiation dose when compared with a mixture of the individual Fab fragments each labelled with Lu-177.

Example 6

Scintigraphy of *Herpes simplex* infection of the eye of a rabbit with the product described in Example 2, labelled with ^{111}In -Fab1-c9-Fab2

25 Corneal de-epithelialisation of one of the eyeballs was performed on adult albino rabbits weighing 3 kg, after topical anaesthesia with naropin. The virus was then inoculated by instillation into the conjunctival sac of the damaged eye during 180 min of 100 to 150 μL of a solution containing 1×10^6

plaque-forming units of clinically isolated *Herpes Simplex* Virus type 1 (HSV-1). Keratitis in the form of a dendritic ulcer was clinically manifest in all the animals after 36 to 48 h. The animals were clinically monitored thereafter, with daily ophthalmological examinations for 2 weeks. No complications were
5 observed in any of the animals.

A portable gamma chamber with high spatial resolution was used for the scintigraphic evaluation. Compound ^{111}In -Fab1-c9-Fab2 prepared according to Example 4 at the dose of 8 $\mu\text{g/kg}$ of body weight was administered 48 h after the infection; scintigraphic evaluation was performed
10 3, 6, 24 and 48 h after administration. The animals were then sacrificed, and both eyeballs were removed.

In all three rabbits studied, the radioactivity of the diseased eye proved to be about 8 times stronger than that of the healthy eye; the greatest difference in enhancement was demonstrated by the measurements taken after
15 3 and 6 h, whereas the contrastographic differences proved lower in the measurements taken after 24 and 48 h.

This *in vivo* test demonstrated that ^{111}In -Fab1-c9-Fab has suitable characteristics to visualise herpes infections. It also demonstrated that the presence of the second recognition molecule, tetanus anti-toxin, in the same
20 conjugate, does not prevent anti-herpetic functionality.

Example 7

Assay of tetanus anti-toxin activity for the product Fab1-c9-Fab2.

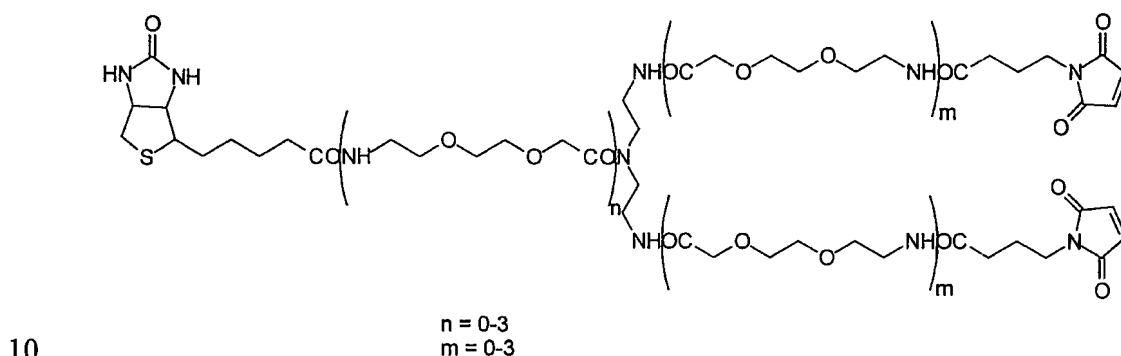
Tetanus anti-toxin activity was determined with a commercial ELISA kit (Tetanus ELISA IgG kit, ICN Diagnostic) in 96-well plates, the secondary
25 antibody being replaced with a Fab human antibody conjugated with horseradish peroxidase (Pierce), and visualised with TMB colorimetric substrate (Sigma). The activity of the product Fab1-c9-Fab2 proved equal to that of Fab isolated from the preparation of starting antibodies (Tetabulin,

Baxter), analysed at equivalent molarities (molecular weight: about 49,000 for the isolated Fab and about 100,000 for Fab1-c9-Fab2).

This *in vitro* test demonstrates that the functionality of both recognition molecules is maintained after conjugation, without any substantial interference
5 between them.

Example 8

Synthesis of a biotin-substituted bis-maleimide compound (compound B)



The compound having the formula shown above, with $m=n=1$ (compound **B**), was prepared from 1,7-bis(trifluoroacetyl)-1,4,7-triazaheptane (prepared according to *US 5,514,810*) by coupling with *N*-*t*-butoxycarbonyl-8-amino-3,6-dioxaoctanoic acid (*Org. Prep. Proced. Int.* 2002, 34, 326-331) in the presence of *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) in DMF. The product obtained was deprotected with K_2CO_3 in MeOH/ H_2O , and the diamine obtained was condensed to *N*-fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic acid using HBTU in DMF. This product was deprotected with piperidine to give the corresponding diamine, which was reacted with 2 molar equivalents of 4-maleimidobutyric acid *N*-hydroxysuccinimidyl ester. The product obtained was deprotected with CF_3COOH and then reacted with biotin *N*-hydroxysuccinimidyl ester to give the end product, **B**.

Example 9

Conjugate with two different anti-mutated E-cadherin Fab fragments bearing a biotin residue (Fab1-B-Fab2)

With preparation methods analogous to those described in Example 2,
5 but using compound B instead of compound 9, a product with a biotinyl residue, called Fab1-B-Fab2, is obtained. This compound can be used for the detection and treatment of lesions according to US5482698.

Example 10

Preparation of a recombinant fusion protein between Fab and a 10 fragment of Pseudomonas exotoxin, Toxin-Fab1.

The plasmid used to produce the anti-*Herpes simplex* human Fab described in example 2 contains cistrons for the heavy chain and the light chain under the control of two identical promoters, from 5' and 3' respectively. Following the method described in US 6,099,842 and using
15 normal genetic engineering techniques, a codifying sequence for a fragment of Pseudomonas exotoxin with a molecular weight of 40,000, called PE40, is inserted into the described plasmid contiguously with the end of the gene codifying the light chain. The modified plasmid serves to produce a recombinant fusion protein between the original Fab, Fab1, and the toxin
20 fragment PE40 in *E. coli*; this construct is called Toxin-Fab1.

Example 11

Preparation of a conjugate between a fusion protein incorporating a Fab and an exotoxin fragment (Toxin-Fab1) and a Fab of other specificity, Toxin-Fab1-c9-Fab2.

25 A conjugate between Toxin-Fab1 and a normal Fab, Fab2, with different specificity from Toxin-Fab, is prepared according to example 2 to obtain a product called Toxin-Fab1-c9-Fab2. As the fusion of PE40 in the carboxy-terminal position of the light chain can leave the affinity of the binding site of

an antibody for the target site intact (US 6,099,842), Toxin-Fab1-c9-Fab2 will continue to recognise cells infected by *Herpes simplex* and cause their death.

Example 12

Preparation of a conjugate between a first Fab specific for a mutation of E-cadherin and fused with a toxin (Toxin-Fab1), and a second Fab specific for a second mutation of E-cadherin.

By following example 10 and using the system employed by Becker et al. (Poster #648, Molecular Targets and Cancer Therapeutics. Miami Beach, Florida, Oct. 29-Nov. 2, 2001) to produce the two different anti-mutant E-cadherin Fab in *E. coli*, a recombinant fusion protein is obtained between Fab specific for the E-cadherin mutated in exon 8 (Fab3) and a fragment of *Pseudomonas* exotoxin, PE40, called Toxin-Fab3. By following the procedures described in example 11, but using Toxin-Fab3 and the Fab anti-E-cadherin mutated in exon 9 (Fab4), a conjugate called Toxin-Fab3-c9-Fab4 is obtained. This product promises to be useful to treat patients with stomach carcinoma characterised by deletion mutations in either exon 8 or in exon 9 of E-cadherin, these mutated E-cadherins not occurring simultaneously in individual patients. In a patient bearing a tumor with a deletion in exon 8 of E-cadherin, the presence of a recognition molecule for E-cadherin with a deletion in exon 9 in the targeted therapeutic product Toxin-Fab3-c9-Fab4 will produce no toxic extra burden without therapeutic benefit. The single bispecific product Toxin-Fab3-c9-Fab4 will be useful for a larger population of cancer patients than a monospecific product. This reduces development and production costs relative to two separate products.

Example 13

Radiodiagnosis and radiotherapy with the products described in Examples 3 and 5

The primary tumour was removed from a patient with a gastric tumour

of the sporadic diffuse type. Immunohistological tests demonstrated that the tumour exposes an E-cadherin with deletion in exon 9. After administration of the product described in Example 3 labelled with ^{111}In , as in Example 4, scintigraphy reveals the location of the metastasis and the residual primary
5 tumour. The dosimetry required for radioimmunotherapy is obtained at the same time. The assay and the image acquisition time are optimised for the patient's weight. Radioimmunological treatment is performed with the product described in Example 5, in administration regimens optimised in the clinical trials required for registration of the product.

CLAIMS

1. An agent for the diagnosis or treatment of those tumours that in an individual patient expose on the cell surface only a number n smaller than N
5 of N different altered forms that a given protein or glycoprotein of said tumour type can assume in a population of patients, said altered forms of the protein deriving from alterations of a normal form present in healthy tissue, said agent comprising:
 - 10 a. a recognition unit consisting of a conjugate of m recognition molecules, where m is at least 2 and equal or smaller than n , and each recognition molecule is specific for a different altered form of the protein, and,
 - b. at least one unit which supplies a diagnostic signal or therapeutic effect, conjugated with or included in said specific recognition
15 unit.
2. An agent as claimed in claim 1, wherein the recognition molecules are selected from among immunoglobulins or fragments thereof, polypeptides and polysaccharides.
3. An agent as claimed in claim 2, wherein at least one recognition
20 molecules is an Fab, F(ab') or scFv fragments.
4. An agent as claimed in claim 2 or 3, wherein the recognition molecules are conjugated to one another by means of a direct covalent bond or by means of a multipurpose linker able to form covalent bonds with the molecules, and/or as a result of the expression of fused genes with suitable linker regions.
- 25 5. An agent as claimed in any one of claims 1-4, wherein at least one of the specific recognition molecules recognises a protein altered as a result of one or more mutations.
6. An agent as claimed in any one of claims 1-4, wherein at least one of

the specific recognition molecules recognises a protein altered as a result of post-translational modifications, deficient post-translational modifications, absence of post-translational modifications or partial degradation.

7. An agent as claimed in any one of claims 1-6, wherein one of the
5 specific recognition molecules recognises an E-cadherin with a deletion in exon 8 and another molecule recognises E-cadherin with a deletion in exon 9.

8. An agent as claimed in any one of the preceding claims, wherein the unit able to provide a diagnostic signal or therapeutic effect is linked directly, via an avidin/biotin or streptavidin/biotin system or via a suitable covalent
10 linker to one of the recognition molecules of the recognition unit, or to the linker that holds the recognition molecules together.

9. An agent as claimed in claim 8, wherein the unit able to provide a diagnostic signal or therapeutic effect is conjugated covalently with biotin, and the recognition unit is conjugated covalently with avidin or streptavidin.

15 10. An agent as claimed in claim 8, wherein the unit able to provide a diagnostic signal or therapeutic effect is conjugated covalently with avidin or streptavidin, and the recognition unit is conjugated covalently with biotin.

11. An agent as claimed in any one of the preceding claims, wherein the unit able to provide a diagnostic signal or therapeutic effect is part of the bond
20 between the recognition molecules of the recognition unit.

12. An agent as claimed in any one of the preceding claims, wherein the unit able to provide a diagnostic signal or therapeutic effect is a radioactive halogen, a chelate of an radioactive isotope, a chelate of a paramagnetic metal ion, a stabilized particle of iron oxide, a stabilised microbubble, a fluorescent,
25 phosphorescent or near-infrared radiation-absorbing compound, a cytotoxic compound, a natural or synthetic toxin, or a photodynamic compound able to generate reduced oxygen species or singlet oxygen by irradiation.

13. An agent as claimed in claim 12, wherein the radioactive halogen is

selected from ^{123}I , ^{124}I , ^{125}I , ^{131}I , ^{75}Br , ^{76}Br , ^{77}Br and ^{82}Br .

14. An agent as claimed in claim 12, wherein the radioactive isotope is selected from among $^{99\text{m}}\text{Tc}$, ^{111}In , ^{203}Pb , ^{66}Ga , ^{67}Ga , ^{68}Ga , ^{161}Tb , ^{72}As , $^{113\text{m}}\text{In}$, ^{97}Ru , ^{62}Cu , ^{64}Cu , ^{67}Cu , ^{52}Fe , $^{52\text{m}}\text{Mn}$, ^{51}Cr , ^{186}Re , ^{188}Re , ^{77}As , ^{90}Y , ^{169}Er , ^{121}Sn ,
5 ^{127}Te , ^{142}Pr , ^{143}Pr , ^{198}Au , ^{199}Au , ^{109}Pd , ^{165}Dy , ^{149}Pm , ^{151}Pm , ^{153}Sm , ^{157}Gd , ^{159}Gd , ^{166}Ho , ^{172}Tm , ^{169}Yb , ^{175}Yb , ^{177}Lu , ^{105}Rh , ^{111}Ag , ^{47}Sc , ^{140}La , ^{211}At , ^{212}Bi , ^{213}Bi , ^{212}Pb , ^{225}Ac , ^{223}Ra , ^{224}Ra and ^{227}Th .

15. An agent as claimed in claim 12, wherein the paramagnetic metal is selected from the metal elements having an atomic number of 21-29, 39, 42,
10 44, 49 or 57-83.

16. An agent as claimed in claim 15, wherein the metal is selected from among Gd^{3+} , Fe^{3+} , Eu^{3+} , Dy^{3+} , La^{3+} , Yb^{3+} and Mn^{2+} .

17. An agent as claimed in claim 15 or 16, wherein the metal or isotope is chelated by chelating groups deriving from diethylenetriamine or from
15 polyamine macrocycles, both substituted by residues bearing carboxy, phosphonic or sulphonic groups.

18. An agent as claimed in any one of claims 1 to 17, wherein the various recognition molecules are conjugated to one another, or said recognition molecules are conjugated with the therapeutic or diagnostic unit, by reaction
20 between sulfhydryl-reactive groups and the sulfhydryl groups present, or generated by reduction of disulfide bridges, on said units/molecules.

19. Pharmaceutical or diagnostic compositions containing an agent as claimed in claims 1-18, in admixture with a suitable vehicle.

20. Compositions as claimed in claim 19, in the form of a kit containing:
25 a. the unit able to provide a diagnostic signal or therapeutic effect, covalently conjugated with biotin, and
b. a recognition unit covalently conjugated with avidin or streptavidin.

21. Compositions as claimed in claim 19, in the form of a kit containing:
- a. the unit able to provide a diagnostic signal or therapeutic effect covalently conjugated with avidin or streptavidin, and
 - b. a recognition unit covalently conjugated with biotin.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/12699

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/00 A61K49/16 A61P35/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 03493 A (UNIV SOUTHAMPTON) 21 March 1991 (1991-03-21)	1-6, 12, 18, 19
Y	the whole document	7-11, 13-17, 20, 21
X	EP 0 404 097 A (BEHRINGWERKE AG) 27 December 1990 (1990-12-27) cited in the application	1, 2, 4-6, 12, 18, 19
Y	page 2, line 1 -page 3, line 46; claims 1-11	1-21
X	WO 93 11161 A (ENZON INC) 10 June 1993 (1993-06-10) cited in the application	1-6, 12, 18, 19
Y	page 4, line 14 -page 7, line 31; claims 1-18 page 13, line 8 -page 17, line 33	1-21
	--- -/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

2 March 2004

Date of mailing of the international search report

11/03/2004

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/12699

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6 447 776 B1 (BECKER KARL-FRIEDRICH ET AL) 10 September 2002 (2002-09-10) cited in the application column 4, line 66 -column 5, line 67; table 2 column 8, line 46 -column 9, line 58 ---	7,8
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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/12699

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	<p>MIEDERER MATTHIAS ET AL: "Comparison of the radiotoxicity of two alpha-particle-emitting immunoconjugates, terbium-149 and bismuth-213, directed against a tumor-specific, exon 9 deleted (d9) E-cadherin adhesion protein." RADIATION RESEARCH, vol. 159, no. 5, May 2003 (2003-05), pages 612-620, XP009026798 ISSN: 0033-7587 (ISSN print) the whole document</p>	1-7, 11, 12, 14
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A	<p>US 5 274 119 A (FRAZIER KEVIN A ET AL) 28 December 1993 (1993-12-28) the whole document</p>	1-21
A	<p>ANELLI ET AL: "L-Glutamic acid and L-lysine as useful building blocks for the preparation of bifunctional DTPA-like ligands" BIOCONJUGATE CHEMISTRY, vol. 10, no. 1, 1999, pages 137-140, ABSTRACT, XP002115959 the whole document</p>	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/12699

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1-2, 4-6, 8-21 (all in parts)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-2,4-6,8-21(all in parts)

Present claims 1, 19, 20 and 21 relate to an agent for the diagnosis or treatment of tumors, whereby the part of the agent corresponding to the recognition unit is defined with respect to the characteristics of the tumor. Said definition is so unclear, and thus represents an undue burden in order to determine what tumors fall under said definition, and consequently what the characteristics of the recognition unit should be. As a result, the claims contain so many options, variables, and possible tumor types that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claim impossible.

Present claim 2 relates to an extremely large number of possible compounds, namely immunoglobulins or fragments thereof, polypeptides and polysaccharides.

Claims 5 and 6 relate to a great number of possible proteins altered as a result of a variety of a mutation. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been carried out for those parts of the application which do appear to be clear (and/or concise), namely those agents recited in the examples, as well as the parts of the agent for the diagnosis or treatment of tumors as defined in claims 3, 7, 8, 12-17.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/12699

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 03/12699

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